REPORT DOCUMENTATION PAGE

Form Approved
OMB NO. 0704-0188

1. AGENCY USE ONLY (Leave Blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	11 June 02	Final Report 15 April 99 – 14 April 02

4. TITLE AND SUBTITLE

Directed Evolution of Novel Enzyme Activities

5. FUNDING NUMBERS

Grant No. N00014-96-1-0340

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9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Office of Naval Research
800 N. Quincy Street
Arlington, VA 22217-5000

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12 a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited.

13. ABSTRACT (Maximum 200 words)

We report here the directed evolution of the two valuable oxidases horseradish peroxidase (HRP) and laccase. We achieved functional expression of HRP in *S. cerevisiae*, and in *E. coli* to an extent that allows the improvement of this important catalyst. Mutagenesis, recombination and screening were successful in increasing total activity 40 fold by improving expression, catalytic activity and thermostability. Mutants were produced at up to 600 U/l/OD in *Pichia pastoris*. We also used computation-guided saturation mutagenesis to create higher active and stabilized HRP mutants more efficiently. We expressed the laccase from *Myceliophthora thermophila* in *Saccharomyces cerevisiae*. We employed directed evolution to improve catalysis as well as expression level to a 170 fold higher total activity. Our evolved yeast mutant has the highest functional expression ever reported for laccase in a nonfungal system. Kinetic characterization of the purified mutants showed that the substrate specificity was left unchanged. The compromised thermostability of the mutants was regained in only one generation of stability screening. For the functional improvement of laccase we developed high throughput assays. PAH bioremediation can be directly assessed with anthracene and for highest sensitivity (0.3 mU/ml) Poly-R, a readily soluble surrogate substrate for PAHs can be used.

14. SUBJECT TERMS	15. NUMBER OF PAGES
laccase, horseradish peroxidase, directed evolution, expression, stability, high throughput screen	Four
	16. PRICE CODE

17. SECURITY CLASSIFICATION OR REPORT	18. SECURITY CLASSIFICATION ON THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	**UNCLASSIFIED**	**UNCLASSIFIED**	**UL**

FINAL REPORT

GRANT #: N00014-96-1-0340

PRINCIPAL INVESTIGATOR: Dr. Frances Arnold

GRANT TITLE: Directed Evolution of Novel Enzyme Activities

INSTITUTION: California Institute of Technology

AWARD PERIOD: 15 April 1999 – 14 April 2002

OBJECTIVE: In this project we developed experimental evolutionary systems to allow protein engineers to improve the oxidative enzymes horseradish peroxidase (HRP) and laccase for Naval and industrial applications.

APPROACH: We use directed evolution (mutagenesis, recombination and high throughput screening) to improve enzymes. Characterization of the molecular changes in evolved mutant enzymes lead to better understanding of the evolutionary process that we use to improve our directed evolution algorithms. When this project began, neither of these key enzymes, HRP or laccase, was expressed in a microbial recombinant host, a prerequisite for directed evolution, and suitable high throughput screens for activity had not been developed. We have obtained, for the first time, functional expression of HRP in *E. coli and* yeasts, and laccase in *S. cerevisiae*. A series of high throughput screening assays are now available for the functional improvement of HRP and laccases towards a variety of properties, including efficient catalysis in the absence of mediators. The HRP/*E. coli* system is currently being used to improve directed evolution strategies through incorporation of appropriate structural information and information from computational methods.

ACCOMPLISHMENTS: **HRP**: We improved the functional expression of HRP in *S. cerevisiae* by mutagenesis, recombination and screening. Total activity was improved 40 fold in *S. cerevisiae* culture supernatants. The best mutants were successfully expressed at up to 600 units/l/OD in *Pichia pastoris* and showed increased specific activity for several substrates. Specific activity was 5.4 fold higher towards ABTS and 2.3 fold higher for guaiacol. Further evolution identified a HRP mutant whose half-life at 60°C was three times that of the wild-type or commercially available HRP. This mutant HRP 13A7-N175S was also more stable in the presence of H_2O_2, denaturating agent (SDS) and at different pH values.

A recombinant expression system in *E.coli* was successfully obtained by cloning the cDNA encoding HRP-C into an expression vector. The recombinant HRP-C is soluble, stable and highly active. In addition, we have established an experimental protocol for measuring HRP-C activity using a colorimetric kinetic assay based on tetramethyl-benzidine (TMB). Computational methods were used to obtain a structural tolerance profile for each amino acid site in the HRP-C sequence by a potential energy function that captures the gross and fine elements governing structural stability. The data

quantitatively describe how mutations affect the three-dimensional structure of HRP-C at every site. We have selected more than 20 sites that are tolerant to mutations for saturation mutagenesis. Screening libraries that contain all possible amino acid substitutions for a third of these sites we found several more active and/or more thermostable mutants.

Laccase: We have been able to express the *Myceliophthora thermophila* laccase (MtL) gene in a protease-deficient strain of *S. cerevisiae* (BJ5465) that is generally suitable for secretion. BJ5465 excreted a low but measurable laccase activity. Ten generations of mutagenesis and recombination were performed using different error-prone PCR conditions for mutagenesis (Taq, mutazyme) and StEP recombination as well as *in vivo* shuffling. PCR and *in vivo* gap repair were used to recombine critical mutations site directly. A high throughput screen with a coefficient of variation (cv) below 10% was developed and laccase activity was assessed with a highly sensitive ABTS based assay. The total activity level of MtL was improved 170 fold.

After nine generations of evolution to improve total activity, the stability of the mutants was significantly reduced. The highest temperature at which the enzyme retained full activity was reduced by 10°C. A stability screen introduced in generation 9 yielded a stability mutant that regained the stability of the wild-type. One generation of stability screening was sufficient to restore this characteristic property of MtL after it was allowed to drift for 9 generations. Characterization of wild-type expressed in yeast in comparison with the wild-type expressed in fungus (NOVO Nordisk) showed that the yeast produced a kinetically damaged enzyme with catalytic efficiencies (kcat/KM) only at 7 to 17% of the fungal enzyme for the substrates tested. Our evolution restored the function of the yeast enzyme to the level of the fungal enzyme and even higher. The improvement was not biased towards the conversion of the substrate used in our screen. Kinetics on ABTS and syringaldazine, a typical phenolic laccase substrate showed a 17 fold improvement in kcat for syringaldazine versus a 13 fold increase for ABTS. The improvement of the total activity was almost equally divided between higher expression and specific activity.

Laccases which work in the absence of mediators will have applications in PAH (polycyclic aromatic hydrocarbon) bioremediation, pulp bleaching and in biofuel cells. Optimization of laccases for mediated applications or independence of mediators using directed evolution requires mediator dependent screens. PAHs are highly toxic organic pollutants. Two colorimetric assays for laccase-catalyzed degradation of PAHs were developed based on studies of the oxidation of twelve aromatic hydrocarbons by fungal laccases from *Trametes versicolor* and *Myceliophthora thermophila*. Using a sodium borohydride water soluble solution (SWS), we could reduce the single product of laccase-catalyzed anthracene oxidation into the orange-colored 9,10-anthrahydroquinone. The sensitivity limit of this colorimetric assay is 25-50 μM of anthraquinone. The limit of detection for laccase units is ~0.01 ABTS Units/ml. An assay utilizing polymeric dye (Poly R-478) as a surrogate substrate in the presence of mediator has been developed as well. The decolorization of Poly R-478 was correlated to the oxidation of PAHs mediated by laccases. The detection limits for laccase are 0.0003 and 0.01 ABTS-units/ml with and without HBT, respectively. This demonstrates that a ligninolytic indicator such as Poly R-478 can be used to screen for PAH-degrading laccases. Poly R-478 is stable and readily soluble. It has a high extinction coefficient and low toxicity toward white rot fungi, yeast, and bacteria, which allow its application in a solid-phase

assay format. We developed another mediator dependent assay that utilizes the mediator dependent oxidation of iodide to iodine by laccase. The sensitivity limit of this assay without mediator is 0.2 U/ml. For the assay including ABTS sensitivity is 0.2 mU/ml.

CONCLUSIONS and SIGNIFICANCE: We achieved functional expression of HRP in yeast and *E. coli*, and have increased catalytic activity and thermostability using directed evolution. Laccase has been expressed in yeast at the highest level ever reported for laccase in a nonfungal system. Catalysis as well as expression level were increased to a 170 fold higher total activity. Thermostability of the mutants was regained in only one generation of stability screening. We developed several assays for mediated and unmediated activity of laccase. With these systems researchers can optimize these important enzymes for desired performance in a wide variety of applications. These systems are available to academic and government laboratories and have been provided to more than a dozen.

PATENT INFORMATION:
Filed US patent application "Expression of Functional Eukaryotic Proteins," Ser. No. 09/247,232, February 9, 1999. Continuation-in-part filed March 27, 2000.

AWARD INFORMATION:
(F.H.A) National Academy of Engineering (2000)
(F.H.A) Professional Progress Award of the American Institute of Chemical Engineers (2000)
(F.H.A) Awarded chaired professorship: Dick and Barbara Dickinson Professor of Chemical Engineering and Biochemistry (2000)
(F.H.A) Fellow, American Institute for Medical and Biological Engineering (2001)

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ABSTRACTS:

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Alcalde, M., Bulter, T. and Arnold, F.H. Oxidation of polycyclic aromatic hydrocarbons (PAHs) by fungal laccases and their relationship with polymeric dyes for biodegradation screening assays. Poster on the First International Conference in Industrial Applications of Biocatalysis San Diego, March 2002.

Bulter, T., Alcalde, M., Sieber, V. and Arnold, F.H. Functional expression of laccase from *Myceliophthora thermophila* in *S. cerevisiae* by directed evolution. First International Conference in Industrial Applications of Biocatalysis San Diego, March 2002.